

Suppression of E-Cadherin Function Drives the Early Stages of Ras-Induced Squamous Cell Carcinoma through Upregulation of FAK and Src

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Advanced stages of epithelial carcinogenesis involve the loss of intercellular adhesion, but it remains unclear how proteins that regulate alterations in cell–cell and cell–matrix adhesion are deregulated to promote the early stages of cancer development. To address this, a three-dimensional human tissue model that mimics the incipient stages of squamous cell carcinoma (SCC) was used to study how E-cadherin suppression promotes tumor progression in Ras-expressing human keratinocytes. We found that E-cadherin suppression triggered elevated mRNA and protein expression levels of focal adhesion kinase (FAK), and increased FAK and Src activities above the level seen in Ras-expressing E-cadherin-competent keratinocytes. The short hairpin RNA (shRNA)-mediated depletion of FAK and Src restored E-cadherin expression levels by increasing its stability in the membrane, and blocked tumor cell invasion in tissues. Surface transplantation of these tissues to mice resulted in reversion of the tumor phenotype to low-grade tumor islands in contrast to control tissues that manifested an aggressive, high-grade SCC. These findings suggest that the tumor-promoting effect of E-cadherin suppression, a common event in SCC development, is exacerbated by enhanced E-cadherin degradation induced by elevated FAK and Src activities. Furthermore, they imply that targeting FAK or Src in human epithelial cells with neoplastic potential may inhibit the early stages of SCC.

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INTRODUCTION

Squamous cell carcinoma (SCC) is initiated as a foci of cells with neoplastic potential that selectively undergo intraepithelial expansion, degrade the basement membrane, and invade into the stroma in the transition from precancer to malignancy (Dlugosz *et al.*, 2002). Although altered tissue structure linked to loss of intercellular adhesion is a morphologic hallmark of the intraepithelial stage of SCC (Bissell and Radisky, 2001), the cellular events that promote the conversion of a premalignant lesion to carcinoma, and control its biological potential, are not well understood.

Signals directed by cadherin-mediated cell–cell adhesions and integrin-mediated cell–matrix interactions regulate normal homeostasis of stratified squamous epithelia, whereas perturbations of intercellular contacts can alter cell motility, survival, and growth. E-cadherin that integrates cell–cell adhesion in adherens junctions with mitogenic signaling is known to play a critical role as a suppressor of invasiveness (Cavallaro and Christofori, 2004; Yap *et al.*, 2007). Loss of E-cadherin function is most commonly associated with advanced stages of SCC progression (Birchmeier and Behrens, 1994; Behrens, 1999; Conacci-Sorrell *et al.*, 2002), and is linked to metastasis (Bissell and Radisky, 2001; Cavallaro and Christofori, 2004) and poor clinical prognosis (Bagutti *et al.*, 1998). Integrins that link matrix components to the cytoskeleton in focal adhesions transmit signals between the cells and the extracellular matrix to regulate cell cycle, motility, and survival in normal and tumorigenic cell contexts (Chen and Gumbiner, 2006). However, it remains unclear how E-cadherin-mediated cell–cell and integrin-mediated cell–matrix adhesions work in concert to modulate and promote the early stages of SCC development.

Focal adhesion kinase (FAK) and Src kinase are linked to cadherins, growth factor receptors, and integrin-associated signaling complexes (Playford and Schaller, 2004; Mitra and

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Abbreviations: 3D, three-dimensional; FAK, focal adhesion kinase; SCC, squamous cell carcinoma; sh-RNA, short hairpin RNA

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Schlaepfer, 2006; Zhao and Guan, 2009). FAK recruitment to focal adhesions results in FAK autophosphorylation and, consequently, activation of Src (Sieg *et al.*, 2000; Roskoski, 2005). FAK hyperphosphorylation by Src directs focal adhesion turnover and cell motility (Schlaepfer *et al.*, 2004; Mitra *et al.*, 2005; Zhao and Guan, 2009). Src can also increase cell motility by phosphorylating cadherin-catenin complexes and regulators of adherens junctions, resulting in E-cadherin internalization and cell spreading (Avizienyte *et al.*, 2002; Fujita *et al.*, 2002; Frame, 2004). Upregulation of FAK and Src has been correlated with the progression of a variety of human cancers. Elevated FAK expression and activity has been linked to breast (Oktay *et al.*, 2003), colon (Withers *et al.*, 1996), prostate (Tremblay *et al.*, 1996), and oral (Schneider *et al.*, 2002) cancers, whereas increased Src activity has been shown in bladder carcinoma (Boyer *et al.*, 2002), head and neck (Mandal *et al.*, 2008), colon (Aligayer *et al.*, 2002), and breast (Reissig *et al.*, 2001) cancers. Nevertheless, how FAK and Src regulate the early stages of SCC remains unclear.

This study provides new insights into the functional consequences of altered E-cadherin-mediated cell-cell adhesion in the early stages of SCC development. In particular, we found that tumor progression in a human three-dimensional (3D) tissue model of SCC induced by oncogenic Ras and E-cadherin suppression was associated with and required the upregulation of FAK and Src activities that magnify E-cadherin loss by increasing its turnover. Furthermore, preventing the increase in FAK and Src activity associated with loss of E-cadherin led to a marked decrease in tumor growth and reverted tumor phenotype from high- to

low-grade carcinoma *in vivo*. These findings demonstrate a critical mechanism by which E-cadherin loss drives the early stages of SCC development through enhanced FAK and Src kinase activities.

RESULTS

E-cadherin suppression in Ras-transformed keratinocytes is associated with elevated FAK expression and FAK and Src tyrosine phosphorylation that promote enhanced cell motility

To better understand how E-cadherin suppression and FAK and Src activities are related during early stages of SCC development, we studied FAK and Src function when Ras-expressing HaCaT-II-4 keratinocytes (II-4 cells) are converted to invasive cells (H-2K^d-Ecad-II-4 cells). This occurs by an overexpression of a dominant-negative E-cadherin fusion protein (H-2K^d-Ecad), which through cytoplasmic sequestration of β -catenin leads to destabilization of E-cadherin- β -catenin complexes and the loss of cadherin-mediated intercellular adhesion (Zhang *et al.*, 2006). As elevated FAK activity is linked to increased phosphorylation at its autophosphorylation site (Tyr397), activation loop (Tyr576/577), and FAT domain (Tyr925; Katz *et al.*, 2003), we compared FAK expression and phosphorylation in both cell lines. Immunoblotting revealed that, compared with non-invasive II-4 cells, invasive H-2K^d-Ecad-II-4 cells showed an approximately twofold increase in FAK expression and elevated FAK phosphorylation at tyrosine Tyr397, Tyr576/577, and Tyr925 (Figure 1a). Furthermore, quantitative real-time reverse transcriptase PCR showed that the increase in FAK expression was associated with a significant upregulation of FAK mRNA level (Figure 1b). Next, we explored whether the increase

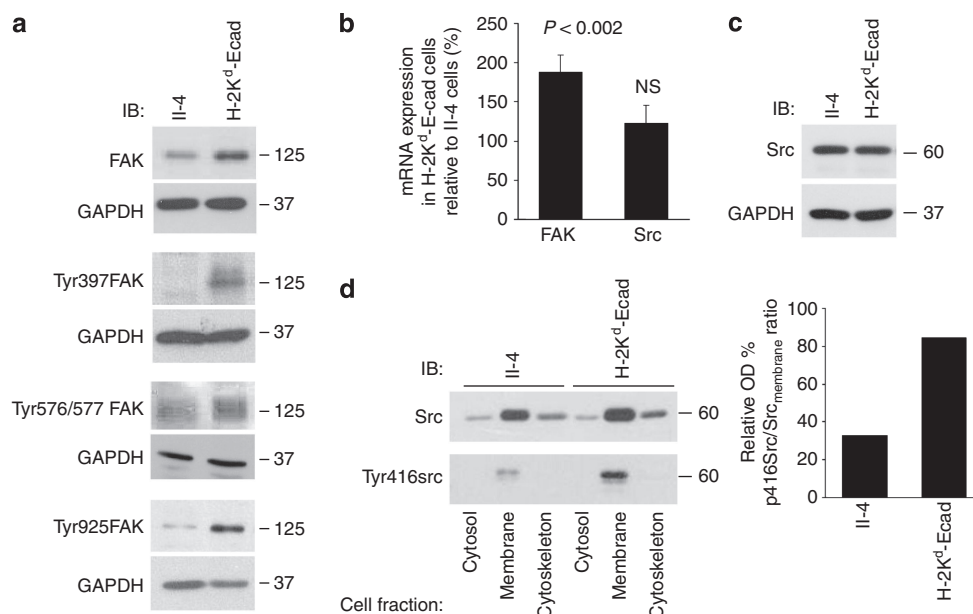


Figure 1. Loss of E-cadherin function is associated with upregulation of focal adhesion kinase (FAK) and Src expression and/or phosphorylation. (a) Western blot (WB) analysis of lysates of H-2K^d-Ecad-II-4 cells revealed an increase in the expression and specific tyrosine phosphorylation of FAK. (b) Quantitative real-time reverse transcriptase-PCR (qRT-PCR) analysis revealed a significant increase in FAK mRNA expression in H-2K^d-Ecad-II-4, relative to its level in II-4 cells. The mean \pm SD values of FAK or Src mRNA expression levels in H-2K^d-Ecad-II-4 relative to II-4 cells are shown. (c) WB analysis showed similar expression levels of Src protein in both cell lines. (d) WB and ratio analysis of cell fractions of the two cell lines revealed increased tyrosine phosphorylation of Src at Tyr416 in the membrane fraction of H-2K^d-Ecad-II-4 cells. NS, nonsignificant.

in FAK expression and activity was associated with changes in Src kinase. Quantitative real-time reverse transcriptase-PCR did not show an increase in Src mRNA level (Figure 1b), and immunoblotting showed similar expression of Src in both cell lines (Figure 1c). As Src recruitment to focal adhesions is associated with its phosphorylation at Tyr416 (Roskoski, 2005), cell fractionation was performed (Figure 1d). Although Src was detected in all cell fractions, it was highest in the membrane fraction of both cell lines and its phosphorylation at Tyr416 was threefold higher in H-2K^d-Ecad-II-4 cells than in II-4 cells (Figure 1d). Thus, E-cadherin suppression in SCC tumor cells resulted in upregulation of FAK mRNA and protein levels that contributed to increased FAK and Src activities.

To determine whether elevated FAK and Src activities increase the motility of H-2K^d-Ecad-II-4 cells, cell motility was analyzed in scrape-wounded II-4- and H-2K^d-Ecad-II-4 cultures in the presence of the inhibitors Tyrphostin AG1007 or PP2 (Supplementary Figure S1 online). Although augmented cell motility was observed in DMSO-treated H-2K^d-Ecad-II-4 cultures relative to DMSO-treated-II-4 cultures, inhibitor-treated cultures showed inhibition of wound closure (Supplementary Figure S1 online) that was reversible upon inhibitor removal from the media. Although Tyrphostin AG1007 and PP2 are not specific only for FAK and Src, and can affect other kinases, inhibition of the augmented motility of H-2K^d-Ecad-II-4 cells was linked, at least in part, to decreased FAK and Src phosphorylation to levels similar to those found in DMSO-treated II-4 cells (Supplementary Figure S2 online).

Prevention of the elevated activity of FAK or Src blocks augmented motility and invasiveness of H-2K^d-Ecad-II-4 tumor cells *in vitro*, and reverses SCC tumor phenotype *in vivo*

To determine whether upregulation of FAK or Src activity in response to decreased E-cadherin levels is necessary for the invasive phenotype of H-2K^d-Ecad-II-4 cells, we used lentivirus-mediated short hairpin RNA (shRNA) to decrease FAK or Src levels in these cells by generating stable sh-FAK- and sh-Src-H-2K^d-Ecad-II-4 cell lines. Immunoblotting revealed that FAK expression in sh-FAK-H-2K^d-Ecad-II-4 cells and Src expression in sh-Src-H-2K^d-Ecad-II-4 cells were reduced approximately 70 and 50%, respectively, when compared with their levels in sh-Scrambled-H-2K^d-Ecad-II-4 cells (Figure 2a). In contrast, the expression level of another Src family member, Fyn, was similar in these cell lines, demonstrating specific downregulation of Src kinase in sh-Src-H-2K^d-Ecad-II-4 cells (Supplementary Figure S3 online). Next, scrape-wounding assay was performed to compare the effect of FAK and Src shRNA-mediated depletion with the pharmacological inhibition described earlier. In comparison with the wound closure seen in sh-Scrambled-H-2K^d-Ecad-II-4 cultures (Figure 2c), cell motility was inhibited in sh-FAK- or sh-Src-H-2K^d-Ecad-II-4 cultures (Figure 2e and g, respectively) and was similar to that seen in sh-Scrambled-II-4 cultures (Figure 2i). Although sh-Scrambled-H-2K^d-Ecad-II-4 cultures showed an average of 85% of wound closure at 24 hours, wound closure was significantly reduced to 58 and

40% in sh-FAK- or sh-Src-H-2K^d-Ecad-II-4 cultures, respectively (Figure 2j). These findings show that the increase in FAK and Src activities present in H-2K^d-Ecad-II-4 cells are necessary for their augmented motility.

To test the concepts revealed using monolayer cultures in a more physiologically relevant setting, an engineered human skin tissue model was used. This 3D tissue model closely mimics the features of the premalignant stages of SCC in human stratified squamous epithelium (Alt-Holland *et al.*, 2005; Margulis *et al.*, 2005). Tissues generated with sh-Scrambled-H-2K^d-Ecad-II-4 cells showed separation between epithelial cells (Figure 3a, arrows) and tumor cells that invaded into the underlying matrix (Figure 3a, arrowheads). In contrast, tissues harboring sh-FAK- (Figure 3b) or sh-Src-H-2K^d-Ecad-II-4 cells (Figure 3c) revealed a greater degree of basal cell layer organization and a significant decrease in tumor cell invasion into the matrix (Figure 3d). These results suggested that, upon E-cadherin loss, increased FAK and Src activities in H-2K^d-Ecad-II-4 cells, above the level seen in II-4 cells, is necessary for their invasive behavior in 3D tissues.

To determine how preventing the increase in FAK and Src activities induced by E-cadherin loss might alter tumor progression *in vivo*, tissues harboring H-2K^d-Ecad-II-4 cells, sh-Scrambled-, sh-FAK-, or sh-Src-H-2K^d-Ecad-II-4 cells were transplanted to the dorsum of nude mice. After 4 weeks, grafts harboring H-2K^d-Ecad-II-4 cells (Figure 3e) or sh-Scrambled-H-2K^d-Ecad-II-4 cells (Figure 3f) demonstrated large erythematous tumors with raised irregular borders and central areas of ulceration. In contrast, grafts harboring sh-FAK-H-2K^d-Ecad-II-4 cells (Figure 3g) slowly enlarged, whereas sh-Src-H-2K^d-Ecad-II-4 tumors remained as small exophytic nodular tumors (Figure 3h). Tumor volume measurements showed that H-2K^d-Ecad-II-4 or sh-Scrambled-H-2K^d-Ecad-II-4 tumors had an approximately fourfold increase in their volume when compared with sh-FAK- or sh-Src-H-2K^d-Ecad-II-4 tumors (Figure 3i). Tumor growth curves showed accelerated development of H-2K^d-Ecad-II-4 and sh-Scrambled-H-2K^d-Ecad-II-4 tumors compared with slower growth of sh-FAK- and sh-Src-H-2K^d-Ecad-II-4 tumors (Figure 3j).

Next, tumor histology was analyzed by hematoxylin and eosin staining, whereas tumor cell proliferation and the degree of tumor differentiation were determined using anti-Ki67 or anti-cytokeratin K1 antibodies, respectively. H-2K^d-Ecad-II-4 (Figure 4a and e) or sh-Scrambled-H-2K^d-Ecad-II-4 (Figure 4b and f) tumors revealed sheets of pleomorphic poorly differentiated cells, areas of widened intercellular gaps (Figure 4f, arrows), and cells that had separated from the tumor edge and infiltrated into the surrounding stroma (Figure 4e, arrows). This aggressive pattern of invasion was associated with the presence of Ki-67-positive tumor cells throughout the tumor cell masses (Figure 4i and j), and the absence of K1 (Figure 4m and n) indicated lack of cell differentiation in these tumors. In contrast, sh-FAK-H-2K^d-Ecad-II-4 tumors showed tumor cell sheets with well-defined margins opposing the stroma (Figure 4c and g, arrowhead). An increased degree of differentiation was evidenced by keratin pearls (Figure 4g, arrow), a more limited distribution

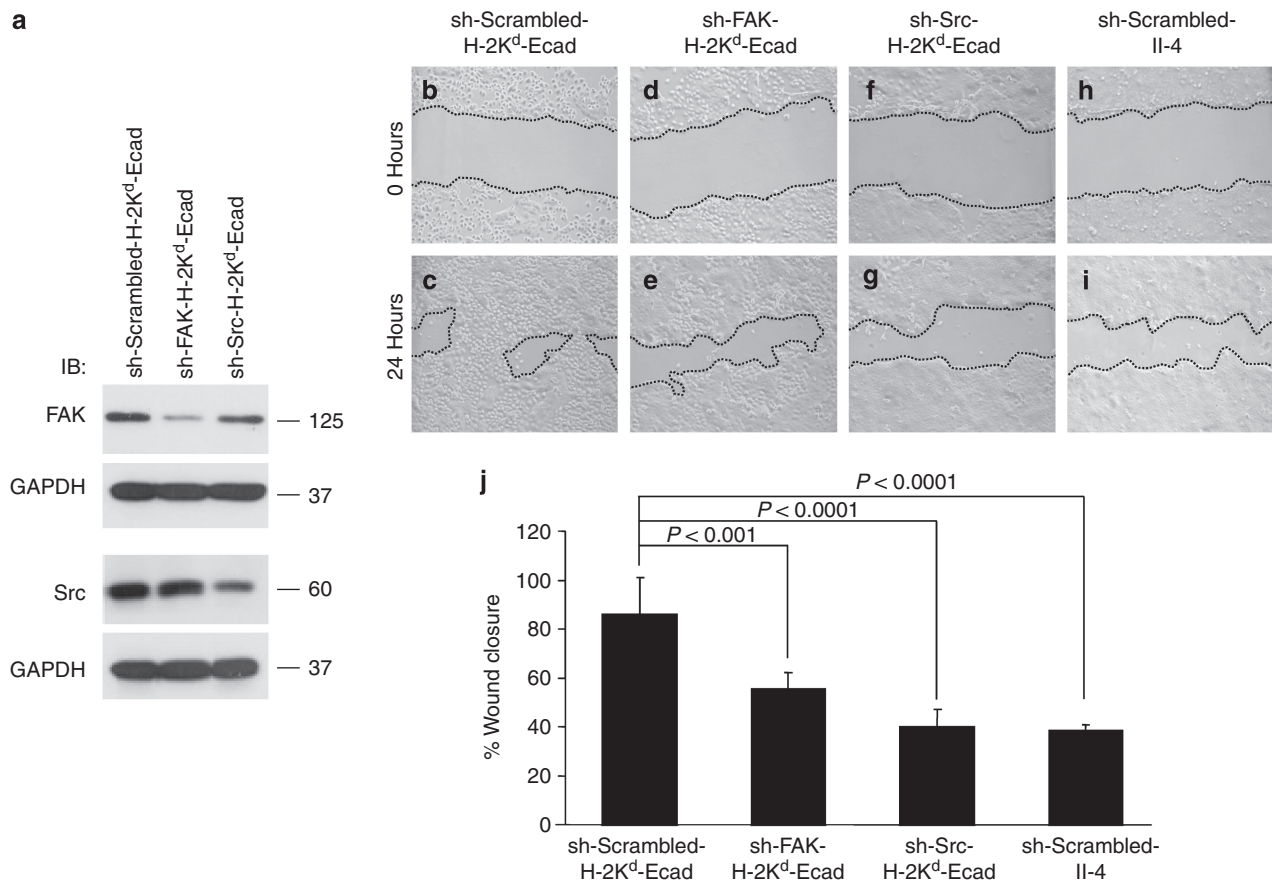


Figure 2. Decreased focal adhesion kinase (FAK) or Src expression in H-2K^d-Ecad-II-4 cells is associated with inhibition of tumor cell motility. (a) Western blot (WB) analysis of lysates of sh-FAK- and sh-Src-H-2K^d-Ecad-II-4 cells showed a decrease in FAK or Src levels, respectively, when compared with their levels in sh-Scrambled-H-2K^d-Ecad-II-4 cells. Scrape-wounded cultures of (b, c) sh-Scrambled-, (d, e) sh-FAK-, and (f, g) sh-Src-H-2K^d-Ecad-II-4 cells and of (h, i) sh-Scrambled-II-4 cells were imaged at 0 and 24 hours following injury. Lines indicate the wound gaps. (j) The mean \pm SD values of wound closure for each cell line are indicated and show the average change in the gaps at 24 hours relative to time 0. The P -values indicate significant changes in wound closure of sh-Scrambled-II-4, sh-FAK-, and sh-Src-H-2K^d-Ecad-II-4 cells in comparison with sh-Scrambled-H-2K^d-Ecad-II-4 cells.

of Ki-67-positive cells to the periphery of the tumors (Figure 4k), and scattered K1 staining in the inner area of the tumors (Figure 4o). sh-Src-H-2K^d-Ecad-II-4 tumors were affected to a greater degree as they revealed a switch to low-grade tumors showing demarcated, well-differentiated tumor islands with defined margins and central masses of keratin (Figure 4d and h, arrows). Ki-67-positive tumor cells were restricted to a basal position at the periphery of these tumor islands (Figure 4l), associated with a strata-specific K1 staining (Figure 4p, arrows). This tumor phenotype was similar to that described previously for E-cadherin-competent II-4 tumors (Margulis *et al.*, 2005, 2006). These data show that E-cadherin suppression in Ras-expressing keratinocytes drives SCC development toward an aggressive tumor phenotype, at least in part, through upregulation of FAK and of Src that, in turn, promote tumorigenesis.

Prevention of the elevated activity of FAK or Src in H-2K^d-Ecad-II-4 cells restores E-cadherin expression and its stability in the plasma membrane

To reveal how prevention of the increased FAK and Src activities reverses the tumorigenic potential of H-2K^d-Ecad-II-4

cells, immunofluorescence analysis of E-cadherin in tissue sections was performed. sh-Scrambled-H-2K^d-Ecad-II-4 tissues showed a faint pattern of E-cadherin staining and cell invasion into the underlying matrix (Figure 5a, arrow, inset). Whereas K1 was absent (Figure 5d), Ki-67-positive cells were seen throughout the epidermis (Figure 5g, arrowheads). In contrast, tissues harboring sh-FAK-H-2K^d-Ecad-II-4 cells showed increased E-cadherin staining at cell-cell borders (Figure 5b). K1 expression was found in suprabasal layers of the epidermis (Figure 5e), and proliferating Ki67-positive cells were more restricted to the basal and immediate suprabasal layers (Figure 5h), suggesting normalization of tissue phenotype. A more significant effect was seen in sh-Src-H-2K^d-Ecad-II-4 tissues that demonstrated restoration of E-cadherin localization at cell-cell borders (Figure 5c), increased K1 expression (Figure 5f), and restricted cell proliferation to the basal layer of the epidermis (Figure 5i). These findings link FAK and Src to the capacity of H-2K^d-Ecad-II-4 cells to acquire invasive properties and to undergo a transition from non-invasive to invasive tumor cell phenotype in 3D tissues. They also show that downregulation of FAK or Src in invasive epithelial tumor cells can block this process in 3D tissues by

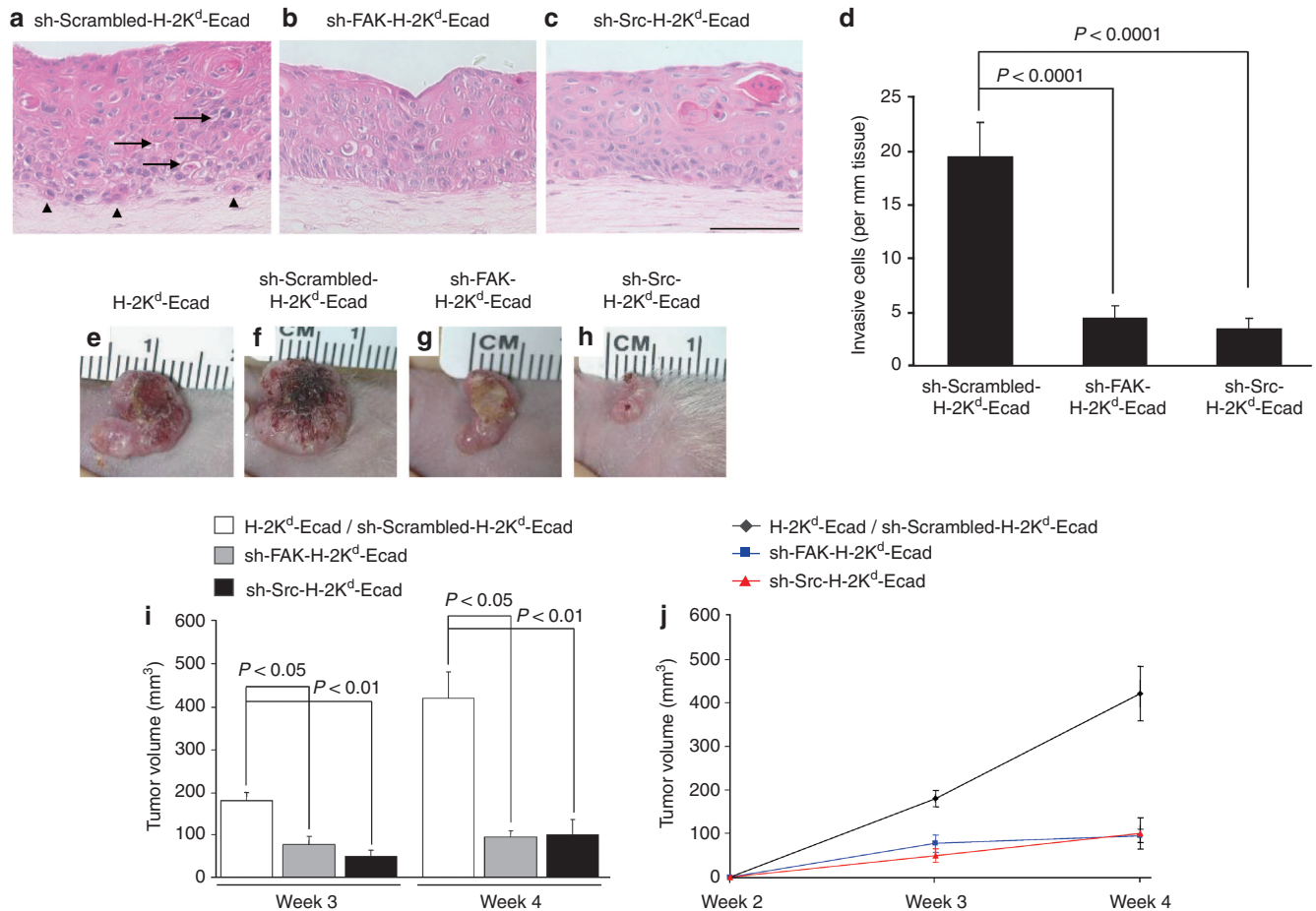


Figure 3. Focal adhesion kinase (FAK) or Src downregulation in H-2K^d-Ecad-II-4 cells is linked to inhibition of tumor cell invasion in three-dimensional (3D) tissues and suppression of tumor growth *in vivo*. Hematoxylin and eosin (H&E) staining of (a) sh-Scrambled-, (b) sh-FAK-, and (c) sh-Src-H-2K^d-Ecad-II-4 tissue sections. sh-Scrambled-H-2K^d-Ecad-II-4 tissues showed intracellular gaps (arrows) and cell invasion into the matrix (arrowheads). Bar = 100 μ m. (d) The mean \pm SD values of cell invasion in each of the tissues are indicated. Representative images of the clinical appearance of (e) H-2K^d-Ecad-, (f) sh-Scrambled-H-2K^d-Ecad-, (g) sh-FAK-H-2K^d-Ecad-, and (h) sh-Src-H-2K^d-Ecad-II-4 tumors at week 4. (i) The mean \pm SD values of tumor volumes are shown. (j) Growth curve of tumor volume demonstrated rapid development of H-2K^d-Ecad- and sh-Scrambled-H-2K^d-Ecad-II-4 tumors and growth inhibition of sh-FAK- and sh-Src-H-2K^d-Ecad-II-4 tumors. For each cohort, $n \geq 3$.

restoring a normalized tissue phenotype. These results indicate that the decrease in E-cadherin expression associated with the increase in FAK and Src activities has a critical mechanistic role in directing the fate of H-2K^d-Ecad-II-4 cells and tumor progression in this tissue model system. To further investigate the effect of FAK and Src depletion on E-cadherin in sh-FAK- or sh-Src-H-2K^d-Ecad-II-4 cells, the protein and mRNA levels of E-cadherin in these cells were analyzed. E-cadherin expression was markedly decreased in sh-Scrambled-H-2K^d-Ecad-II-4 cells compared with sh-Scrambled-II-4 cells (Figure 5j). However, shRNA targeting of FAK or of Src in H-2K^d-Ecad-II-4 cells resulted in restoration of E-cadherin expression to the level seen in sh-Scrambled-II-4 cells (Figure 5j), whereas no significant changes in E-cadherin mRNA levels were observed (Figure 5k).

Upon its removal from the cell membrane, E-cadherin is either targeted to degradation or recycled back to the cell membrane (Janes *et al.*, 2008). To elucidate the possible

mechanism underlying the restoration of E-cadherin expression in sh-FAK- or sh-Src-H-2K^d-Ecad-II-4 cells, E-cadherin turnover was assayed by treating cultures with 10 μ M Cycloheximide in order to block *de novo* protein synthesis and to test whether FAK and Src depletion affected E-cadherin stabilization at the plasma membrane. Immunoblotting revealed that, compared with Cycloheximide-treated sh-Scrambled-II-4 cells, Cycloheximide-treated sh-Scrambled-H-2K^d-Ecad-II-4 cells showed an accelerated degradation of E-cadherin over 24 hours (Figure 5l). In contrast, E-cadherin was considerably more stable in Cycloheximide-treated sh-FAK- or sh-Src-H-2K^d-Ecad-II-4 cells, and under these conditions its levels were higher than those of sh-Scrambled-II-4 cells (Figure 5l). Figure 5m illustrates the percentages of the remaining E-cadherin in the Cycloheximide-treated cultures over 24 hours relatively to the corresponding Cycloheximide-treated cultures at time 0. At the 24-hour time point, when compared with the remaining 71% of E-cadherin in sh-Scrambled-II-4 cells,

E-cadherin level in sh-Scrambled-H-2K^d-Ecad-II-4 cells decreased to 41%, whereas its levels in sh-FAK- or sh-Src-H-2K^d-Ecad-II-4 cells were 72 and 70%, respectively. Under these conditions, the exogenous H-2K^d-Ecad fusion protein decreased similarly in sh-Scrambled-, sh-FAK-, and sh-Src-H-2K^d-Ecad-II-4 cell lines (Supplementary Figure S4 online). These data indicate that downregulation of FAK and Src in sh-FAK- and sh-Src-H-2K^d-Ecad-II-4 cells, respectively, contribute at least in part to a decrease in E-cadherin degradation and an increase in E-cadherin stability in these cells.

DISCUSSION

This study reveals that loss of the suppressor of invasiveness, E-cadherin, drives the early stages of Ras-induced SCC progression through increased activation of FAK and Src that, in turn, further directs destabilization of E-cadherin at the plasma membrane, thus enhancing its degradation. Elevated FAK and Src activities magnify the effect of E-cadherin

suppression, promote tumor cell invasion in engineered tissues, and result in progression to an aggressive carcinoma *in vivo*. Preventing FAK or Src upregulation resulted in increased E-cadherin expression by enhancing its stability in cell-cell contacts that, at least in part, blocked the invasiveness of H-2K^d-Ecad-II-4 cells and directed them to a more normalized cell fate in 3D tissues. *In vivo*, these tissues showed reversion of an aggressive high-grade tumor phenotype to a low-grade, indolent tumor behavior. These data suggest a critical new mechanistic role for E-cadherin suppression in directing FAK and Src upregulation as an important component of early SCC development.

We have previously shown that loss of E-cadherin-mediated adhesion in H-2K^d-Ecad-II-4 cells was linked to increased integrin-mediated adhesion (Zhang *et al.*, 2006) and enhanced cell motility (Alt-Holland *et al.*, 2008). This interdependence between loss of cell-cell and gain of cell-matrix adhesions suggested a co-activation of regulatory

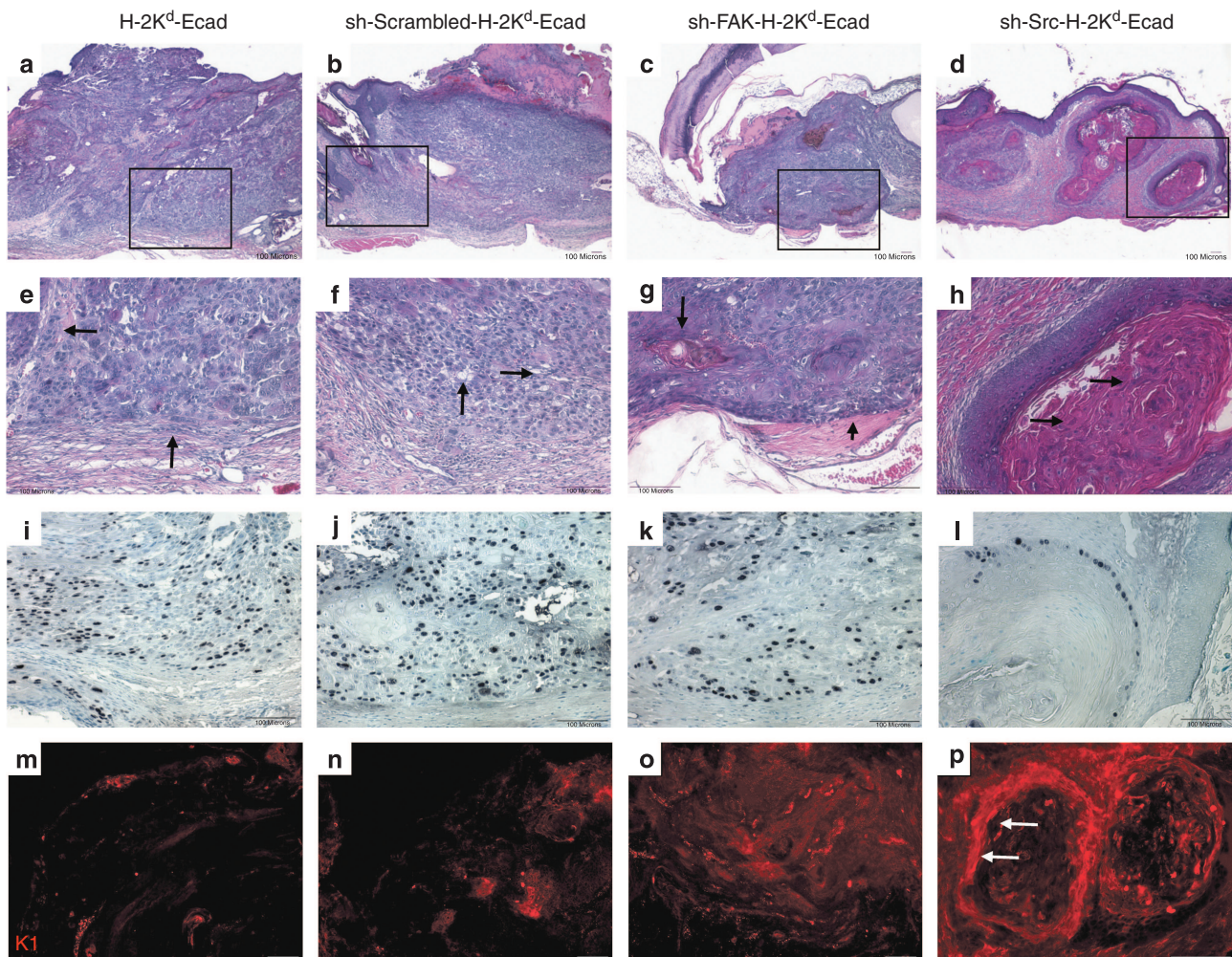
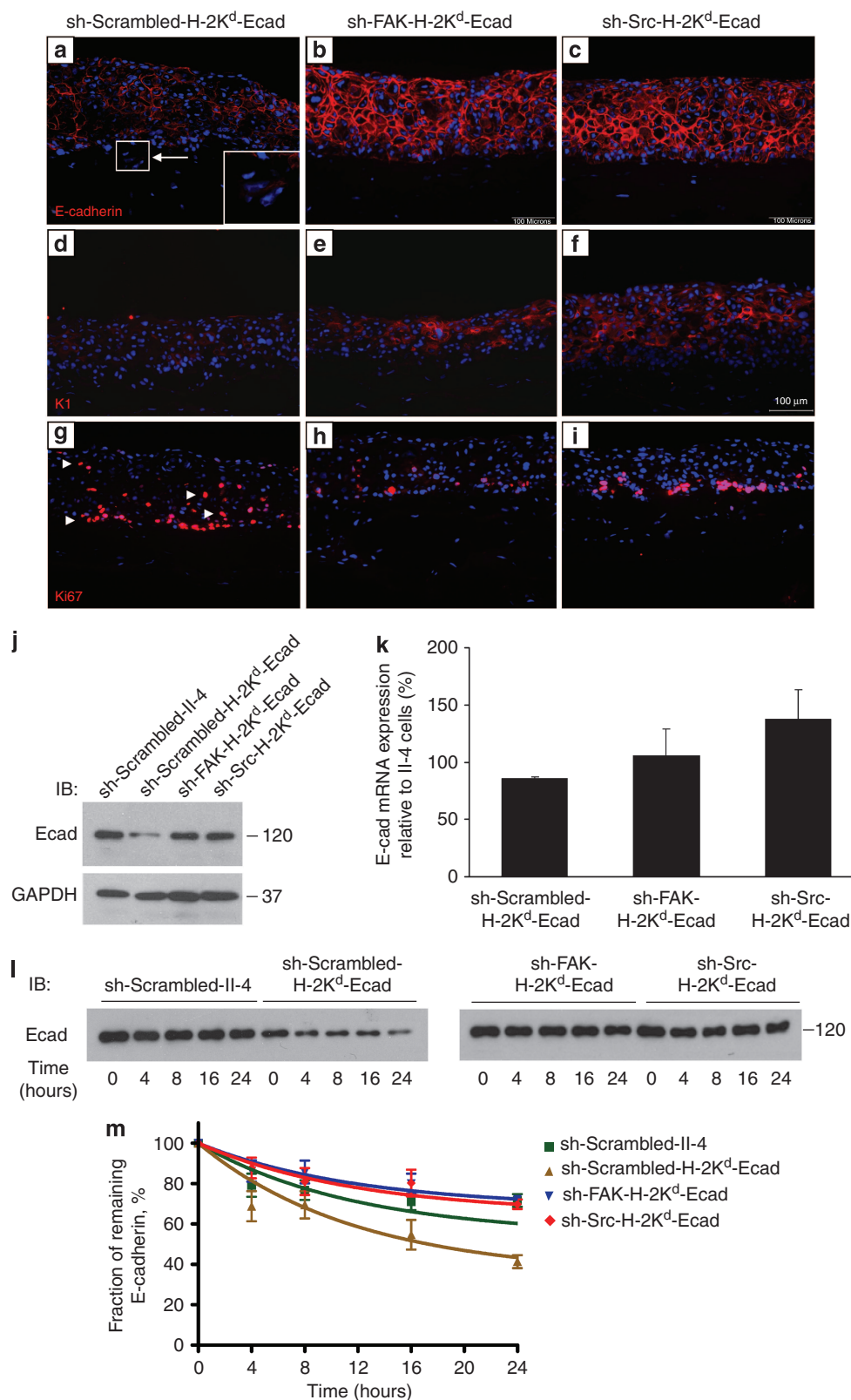


Figure 4. E-cadherin-suppressed H-2K^d-Ecad-II-4 cells exhibit a switch from high-grade to low-grade tumors upon downregulation of focal adhesion kinase (FAK) or Src. Images of (a-d) and in higher magnification (e-h) hematoxylin and eosin (H&E), (i-l) Ki67 immunohistochemistry, and (m-p) K1 immunofluorescence staining of tumor sections. (a, e) H-2K^d-Ecad- and (b, f) sh-Scrambled-H-2K^d-Ecad-II-4 tumors showed (e, f, arrows) poorly differentiated, invasive cells and (i, j) Ki67-positive tumor cells, whereas (m, n) K1 was undetected. (c, g) sh-FAK-H-2K^d-Ecad-II-4 tumors showed (g, arrow) tumor cell differentiation, (o) disperse K1, and (k) Ki67-positive cells that were more limited to the tumor invasive edge. (d, h) Well-differentiated sh-Src-H-2K^d-Ecad-II-4 tumor islands showed (h, arrows) keratin pearls, (p, arrows) stratum-specific K1, and (l) Ki67-positive tumor cells at the periphery of the tumor clusters. Bar = 100 μm.

events that could orchestrate and augment H-2K^d-Ecad-II-4 tumorigenic potential. FAK and Src, which are linked to cadherins and integrins (Mitra and Schlaepfer, 2006; Zhao

and Guan, 2009) and are involved in tumor cell adhesion, migration, and invasion (Summy and Gallick, 2003; Schlaepfer *et al.*, 2004; Siesser and Hanks, 2006), emerged as



candidates to regulate the behavior of these cells. FAK or Src upregulation has been correlated with advanced stages of cancer progression (Jones *et al.*, 2000; Ishizawa and Parsons, 2004). For example, elevated expression and activation of FAK has been linked to the progression of breast, colon, prostate, and oral cancers (Withers *et al.*, 1996; Tremblay *et al.*, 1996; Oktay *et al.*, 2003; Schneider *et al.*, 2002), and amplification of the FAK gene has been shown in invasive SCC (Agochiya *et al.*, 1999). Increased Src activation has been associated with head and neck SCC and breast cancers (Reissig *et al.*, 2001; Mandal *et al.*, 2008). In colon cancer, increased Src expression has been found in preneoplastic lesions and activating mutations are seen in metastatic foci (Irby *et al.*, 1999). In this study, the use of *in vitro* 3D tissues and *in vivo* transplants that closely mimic the features of early stages of SCC in the human skin enabled us to analyze the fate of E-cadherin-suppressed tumor cells in which FAK or Src were either upregulated or suppressed. Here, we report that E-cadherin suppression in the incipient stages of SCC drives the upregulation of FAK mRNA and protein levels and of FAK and Src activities above the level seen in E-cadherin-competent II-4 cells. This increase is necessary for the invasiveness of H-2K^d-Ecad-II-4 cells in 3D tissues, and consequently for the progression of premalignant tissues to aggressive carcinomas *in vivo*. A modest decrease in FAK or Src was sufficient to inhibit the invasiveness of these cells by restoration of E-cadherin at cell-cell borders and normalization of tissue phenotype. This, in turn, resulted in reversion of the aggressive tumor phenotype and inhibition of tumor growth *in vivo*. These data demonstrate the critical regulatory role of elevated FAK and Src activity in exacerbating the tumor-promoting effect of decreased E-cadherin expression during the early stages of SCC development.

How does increased activity of FAK and Src affect the motility of early-stage E-cadherin-suppressed SCC tumor cells? H-2K^d-Ecad-II-4 cells showed increased phosphorylation of the autophosphorylation site of FAK (Tyr397), the activation domain of FAK (Tyr576/577), and FAK C-terminus domain (Tyr925). FAK phosphorylation at Tyr925 has been linked to focal adhesion turnover, cell spreading, and motility (Katz *et al.*, 2003). Thus, E-cadherin suppression in concert with increased FAK activity and FAK phosphorylation on Tyr925 can facilitate, at least in part, the augmented motility of H-2K^d-Ecad-II-4 cells. In fibroblasts, depletion of the β 1 integrin (Raghavan *et al.*, 2003) or of FAK (Ilic *et al.*, 1995) resulted in increased numbers of focal contacts and inhibition of cell motility, whereas stable reconstitution of FAK in FAK-null fibroblasts promoted focal adhesion turnover and enhanced cell motility (Sieg *et al.*, 1999). In carcinoma cells,

increased FAK expression was correlated with enhanced cell motility (Hauck *et al.*, 2001), whereas depletion of FAK in keratinocytes inhibited tumor formation (McLean *et al.*, 2004). Our study has extended these findings to show that E-cadherin loss in H-2K^d-Ecad-II-4 cells led to increased FAK and Src activity that consequently resulted in Src-mediated hyperphosphorylation of FAK, leading to enhanced motility of these cells. In supporting this, exposure of H-2K^d-Ecad-II-4 cells to FAK or Src inhibitors blocked their augmented motility and led to decreased phosphorylation of FAK and Src to a similar level seen in II-4 cells. Nevertheless, these findings do not exclude the possible activation and involvement of other Src family kinases, such as Fyn, in the augmented neoplastic phenotype of H-2K^d-Ecad-II-4 cells (Yadav and Denning, 2011).

How can upregulation of Src and FAK exacerbate E-cadherin loss during the early stages of SCC? It has been shown that overexpression of active Src in the colorectal cancer cell line, KM12C, resulted in disruption of E-cadherin-mediated adhesion that was dependent on Src-mediated FAK phosphorylation (Avizienyte *et al.*, 2002). Our study expands these observations by showing that suppression of E-cadherin function can also promote elevated Src activity and lead to similar consequences of Src overexpression. Active Src has also been shown to induce tyrosine phosphorylation of cadherin-catenin complexes or regulators of adherens junctions, resulting in E-cadherin endocytosis and disruption of cadherin-mediated cell-cell adhesions, thus further enhancing tumor cell motility (Fujita *et al.*, 2002; Irby and Yeatman, 2002; Frame, 2004; Jeanes *et al.*, 2008). However, an alternative possibility to increased E-cadherin endocytosis in H-2K^d-Ecad-II-4 is that degradation and turnover of E-cadherin can also occur via MMP9-mediated E-cadherin ectodomain shedding from the plasma membrane that can be promoted by FAK or Src as seen in ovarian carcinoma cell lines (Cowden Dahl *et al.*, 2008; Symowicz *et al.*, 2008). Our findings suggest that as a result of E-cadherin suppression, elevated Src activity can advance the early stages of SCC by hyperphosphorylation of FAK and by further deregulating E-cadherin-mediated adhesions. The fact that Src suppression limits its accessibility to phosphorylate FAK and to further decrease E-cadherin stability in the plasma membrane can explain, at least in part, why sh-Src-H-2K^d-Ecad-II-4 tissues showed a more profound degree of normalization *in vitro* and a shift to a low-grade behavior *in vivo* compared with sh-FAK-H-2K^d-Ecad-II-4 tissues. The findings that E-cadherin suppression led to simultaneous activation of FAK and Src in H-2K^d-Ecad-II-4 cells suggest that the interaction between both kinases can amplify the tumorigenic potential of

Figure 5. Decreased focal adhesion kinase (FAK) or Src in H-2K^d-Ecad-II-4 cells is associated with upregulation and stabilization of E-cadherin protein leading to normalization of tissue phenotype. Immunofluorescence staining of sh-Scrambled-H-2K^d-Ecad-II-4 tissue sections showed (a, arrow, inset) inconsistent E-cadherin staining and cell invasion, and although (d) K1 was not detected, (g, arrowheads) Ki-67-positive cells appeared throughout the epidermis. sh-FAK-H-2K^d-Ecad-II-4 tissues showed intense (b) E-cadherin staining, (e) K1 expression, and (h) Ki67-positive cells at the basal and immediate suprabasal layers. sh-Src-H-2K^d-Ecad-II-4 tissues revealed (c) intense E-cadherin staining, (f) elevated K1, and (i) Ki67-positive cells at the basal layer. Bar = 100 μ m. (j) Although an increase in E-cadherin protein was detected in sh-FAK- or sh-Src-H-2K^d-Ecad-II-4 cells, (k) E-cadherin mRNA level was not significantly changed. (l) Representative western blot (WB) of E-cadherin in Cycloheximide-treated cultures, and (m) the mean \pm SD of the remaining E-cadherin in these cultures in five independent experiments.

E-cadherin-suppressed tumor cells. They also imply that these kinases may exert complementary roles in regulating tumor cell invasiveness during the early stages of SCC development and the course of progression of this disease.

Collectively, our findings demonstrate an important new role for E-cadherin in the early stages of SCC development. Abrogation of E-cadherin-mediated adhesion in Ras-expressing early-stage human epithelial tumor cells induces elevated expression and/or activation of FAK and Src that work in concert to promote aggressive tumor cell behavior during incipient SCC development. Preventing the increase in FAK and of Src activities profoundly altered tumor outcome *in vivo*. As FAK and Src evolved as therapeutic targets for cancer invasion and metastasis (McLean *et al.*, 2005; Brunton and Frame, 2008; Rucci *et al.*, 2008), our findings suggest that the premalignant stages of SCC development may be inhibited by targeting these kinases in human epithelial cells with neoplastic potential. Moreover, by further understanding the events occurring in the progression of precancer to malignancy in clinically relevant, *in vivo*-like human tissues, new therapeutic approaches designed to block these events may be formulated to impair early cancer invasion, thus preventing SCC development or reoccurrence.

MATERIALS AND METHODS

Cells

Human foreskin fibroblasts were derived from newborn foreskins and grown in DMEM with 10% fetal bovine serum (FBS, HyClone, Thermo Scientific, Rockford, IL). HaCaT-II-4 keratinocytes (Boukamp *et al.*, 1990) were grown in DMEM (Invitrogen, Carlsbad, CA) with 5% fetal bovine serum. H-2K^d-Ecad-II-4 cells were generated by retroviral infection of HaCaT-II-4 cells as described (Margulis *et al.*, 2005). II-4 and H-2K^d-Ecad-II-4 cells were infected with sh-Scrambled-, sh-FAK-, or sh-Src-lentiviruses at a multiplicity of infection between 0.5 and 1. Stable cell lines were selected with Blasticidin (7 µg/ml; Invitrogen). Cells were grown at 37°C in 7.5% CO₂. FAK or Src depletion was confirmed by western blot analysis at the end of the selection process, during cell passages, and before the fabrication of 3D tissues.

Construction of sh-FAK and sh-Src-mediated lentiviruses

Previously published small interfering RNA sequences to knock down FAK (McLean *et al.*, 2004; Tilghman *et al.*, 2005) or Src (Trevino *et al.*, 2006; Wu *et al.*, 2008) were designed into oligonucleotides (IDT, San Diego, CA) with a loop sequence of *tcaagaga*. These oligonucleotides were ligated into hairpin vectors using the BLOCK-iT Vector Kit (Invitrogen) and recombined into pLenti6-DEST (Invitrogen). Lentiviruses expressing shRNA against FAK, Src, or a scrambled sequence were titrated using an end point dilution assay.

3D cell culture

Tissues harboring H-2K^d-Ecad-II-4 cells or sh-Scrambled-, sh-FAK, or sh-Src-H-2K^d-Ecad-II-4 cells were generated as previously described (Margulis *et al.*, 2005). Briefly, 6×10^5 tumor cells were seeded onto human foreskin fibroblast-populated type I collagen gels, in deep-well polycarbonate tissue culture inserts (Organogenesis, Canton, MA). Cultures were maintained submerged for 3 days in low-calcium epidermal growth medium and for 2 days in normal-calcium epidermal growth medium before they were raised to an air-liquid interface for 7 days.

In vivo tissue transplantation

All animal experiments were conducted according to a protocol approved by the Tufts University Institutional Animal Care and Use Committee. Briefly, a 1.3 cm skin section from the dorsum surface of 6-week-old male Swiss nude mice (N:NIHS-nuf DF; Taconic Farms, Germantown, NY) was removed from groups of $n \geq 3$ per cell line, and tissues were transplanted at the excision site. Following bandage removal, tumor volume was estimated using the formula volume = length \times width \times height. Mice were killed 4 weeks after grafting, and tumors were excised and processed as described (Margulis *et al.*, 2006).

Quantification of cell invasion

For each fabricated tissue, events of tumor cell invasion into the matrix were counted and averaged from >100 microscope fields in >20 sections from multiple experiments. Averages represent ≥ 3 independent experiments \pm SD. The *P*-values were calculated using the Mann-Whitney test.

E-cadherin protein turnover

Cell cultures were treated with either 10 µM DMSO (vehicle) or Cycloheximide (Sigma, St Louis, MO) for 0, 4, 8, 16, and 24 hours, and cell lysates were subjected to western blot analysis. Immunoblots of three independent experiments were quantified by densitometry, using Image J software (Java-based image processing program, NIH; <http://rsb.info.nih.gov/ij/>). The percentage of the remaining E-cadherin was measured relative to treatment with DMSO and reported as a value relative to time 0.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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